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Different modes of membrane permeabilization by two RTX toxins: HlyA from *Escherichia coli* and CyaA from *Bordetella pertussis*

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ABSTRACT

This study clarifies the membrane disruption mechanisms of two bacterial RTX toxins: αhemolysin (HlyA) from *Escherichia coli* and a highly homologous adenylate cyclase toxin (CyaA) from *Bordetella pertussis*. For this purpose, we employed a fluorescence requenching method using liposomes (extruded through filters of different pore size — 1000 nm, 400 nm or 100 nm) with encapsulated fluorescent dye/quencher pair ANTS/DPX. We showed that both toxins induced a graded leakage of liposome content with different selectivities α for DPX and ANTS. In contrast to HlyA, CyaA exhibited a higher selectivity for cationic quencher DPX, which increased with vesicle diameter. Large unilamellar vesicles (LUV_{1000}) were found to be more suitable for distinguishing between high α values whereas smaller ones (LUV_{100}) were more appropriate for discriminating an all-or-none leakage ($\alpha = 0$) from the graded leakage with low values of α. While disrupting LUV_{1000}, CyaA caused a highly cation-selective leakage ($\alpha \sim 15$) whereas its mutated form with decreased channel K^+/Cl^- selectivity due to two substitutions in a predicted transmembrane segment (CyaA-E509K + E516K) exhibited much lower selectivity ($\alpha \sim 6$). We concluded that the fluorescence requenching method in combination with different size of liposomes is a valuable tool for characterization of pore-forming toxins and their variants.

1. Introduction

Both adenylate cyclase toxin of *Bordetella pertussis* (CyaA, 177 kDa) and αhemolysin of *Escherichia coli* (HlyA, 117 kDa) belong to the RTX toxin (RTX, Repeat in ToXin) family. Their C-terminal hemolysin parts, RTX cytolysin moieties, are highly homologous [1] (see Fig. 1). RTX cytolysin part is known for its ability to damage biological membranes even without the presence of specific cellular receptors [2–4]. Nevertheless, cellular receptors for both toxins have been discovered [5,6].

RTX repeat domain bears multiple Ca^{2+} binding sites that are necessary for the full hemolytic activity. N-terminal part of the hemolysin is responsible for the binding to biological membranes and channel formation due to its high hydrophobicity and double acylation.

Whereas HlyA is known mainly for its hemolytic capacity, CyaA interacts with its target membrane in a more complex way. After binding to the target cell membrane it translocates its N-terminal adenylate cyclase domain into the cytoplasm where it rapidly converts ATP to cAMP upon activation by cellular calmodulin [7,8], thus causing rapid ATP depletion and cell death [9,10]. In parallel, the RTX hemolysin moiety of CyaA forms small cation-selective membrane channels that allow the entry of monovalent cations and are responsible for osmotic lysis of the cell [11].

In black lipid membranes, CyaA forms very narrow channels with an estimated diameter of 0.6–0.8 nm [12,13] whereas HlyA forms much wider channels (approx. 2–3 nm) with a lifetime much longer than those of CyaA [14]. Both CyaA and HlyA channels are supposed to be formed by oligomers of the toxin subunits [3,11,14–17]. Both toxins were recently shown to induce the formation of non-lamellar phases in biological membranes [18,19].

An "all-or-none" mechanism (see below) by which toxin releases model lipid vesicle content was found for HlyA from *E. coli* [3]. The authors used the method developed by Parente et al. [20] in which liposomes loaded with fluorescence probe ANTS and its quencher DPX were subjected to toxin-induced lysis. The mechanism by which CyaA from *Bordetella pertussis* disrupts a membrane, however, has not yet been investigated.

When liposomes loaded with ANTS/DPX pair are subjected to toxin-induced lysis there are in general two possible different mechanisms of membrane disruption:

1) "Graded leakage" means that all of the vesicles affected by the toxin are losing continuously some part of their inner contents. This corresponds to the formation of transient narrow pores which do not allow one-step release of their inner contents. This release can theoretically be more

Abbreviations: ANTS, 8-Aminonaphthalene-1,3,6-trisulfonic acid; BLM, black lipid membranes; CyaA, adenylate cyclase toxin from *Bordetella pertussis*; DPX, *p*-xylene-bis-pyridinium bromide; FITC, fluorescein-5-isothiocyanate; HlyA, αhemolysin from *Escherichia coli*; LUV_{100}, LUV_{400}, LUV_{1000}, large unilamellar vesicles extruded through filters of 100, 400 and 1000 nm pore-size, respectively; PolyB, Polymyxin B from *Bacillus polymyxa*

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effective either for DPX^+ (selectivity $\alpha > 1$, see Materials and methods) or for $ANTS^-$ ($\alpha < 1$). Usually, a high selectivity of the graded release for cationic DPX^+ was observed [21–24]. Different authors interpreted such data as i) the formation of a cation-selective channel [25] or ii) the formation of temporary "non-channel" disruptions that induced a release of cationic DPX^+ accumulated preferentially near a negatively charged membrane surface [26]. This second explanation might be valid in some cases since high selectivity α is often observed for a leakage by different membrane-disrupting peptides, both cationic and anionic [21–24].

2) "All-or-none leakage" is mostly interpreted as a leakage through a large-diameter pore. Such a pore allows a rapid release of inner vesicle contents by diffusion, within subsecond time [27]. After such a leakage, there are two distinct populations of vesicles — the first represented by completely "empty" vesicles and the second one with the vesicles that remained unaffected by the leakage, having an unchanged concentration of ANTX/DPX inside. The extent of quenching inside the latter vesicle population remained constant because there was no loss of DPX. The all-or-none pathway has been observed for several toxins including HlyA (*E. coli*) [3], magainin 2 (*Xenopus laevis*) [28] and granule cytolysin (*Rattus rattus*) [29]. The all-or-none mechanism shared by all these membrane-perturbing amphipathic peptides suggests that these proteins share some common characteristics like big pore cross-section and its high stability.

The main aim of our work was to compare the homologous bacterial RTX toxins CyaA of *Bordetella pertussis* and HlyA of *Escherichia coli* with respect to the type of the toxin-induced leakage of large unilamellar soybean phosphatidylcholine membrane vesicles (LUVs) containing encapsulated fluorescent dye/quencher pair ANTS/DPX. We used the fluorescence requenching method [21,22] for determining the leakage mechanism. This method has been recently used for characterization of several pore-forming proteins [23,25,26,30–32]. The reason for our CyaA and HlyA comparison was also the fact that these two toxins were never compared under identical conditions using the same liposomal membranes, although several studies were carried out with each of the toxins [2,3,19,33]. In addition we used the CyaA mutant form CyaA-E509K + E516K that was reported to form membrane channels in black lipid membranes with longer lifetime and decreased cation selectivity [15]. One of our goals was to find out if the requenching method was sensitive enough to distinguish between the channel properties of CyaA and CyaA-E509K + E516K.

Together with CyaA and HlyA we used another bacterial toxin, Polymyxin B from *Bacillus polymyxa*, an antibacterial cyclic decapeptide targeted against gram-negative bacteria. The insertion of the heptapeptide ring of Polymyxin (PolyB) into the bilayer was reported to induce fusion of the membranes together with disruption of the membrane integrity that allowed the passage of proteins through the membrane perturbations [34–37]. We expected therefore an "all-or-none" mechanism of the leakage since induced by high doses of PolyB that were used in our experiments.

At the same time, we decided to study the effect of the size of large unilamellar vesicles on an observed mechanism of leakage. Such effect has not yet been studied; it was usually neglected in similar studies, and is expected to be significant mainly for the quantitation of the "all-or-none" mechanism.

2. Material and methods

2.1. Chemicals

8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes, L-α-phosphatidylcholine from soybean, Type II-S (SPC) and FITC was purchased from Sigma. All other chemicals used were of analytical grade.

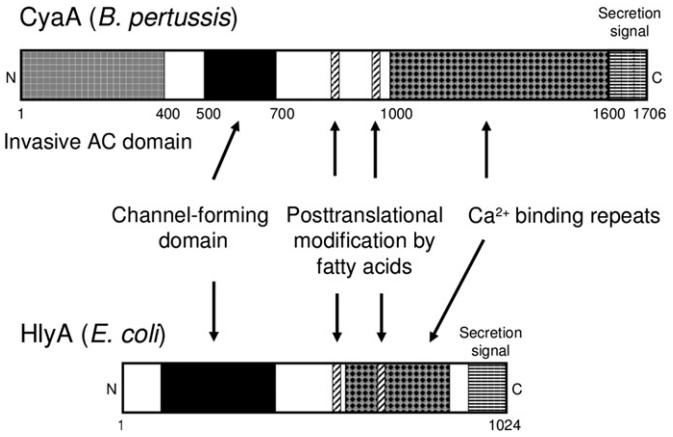

Fig. 1. Schematic representation of CyaA of *Bordetella pertussis* and HlyA of *Escherichia coli*. Both RTX toxins share a highly homologous C-terminal hemolysin part.

Purified recombinant HlyA, CyA and its mutant form CyaA-E509K + E516K were provided by Dr. Peter Šebo and Dr. Radim Osička (Institute of Microbiology, CAS, Czech Republic). Both toxins were stored in 8 M urea at −20 °C. Polymyxin B sulfate (Sigma) has been dissolved in Tris–HCl buffer (pH = 7.4) and used immediately in our experiments.

2.2. Vesicle preparation

The method employed for the preparation of large unilamellar vesicles (LUV) represents a further modification of that published before in [38]. SPC (15 mg) was dissolved in 1 ml of chloroform that was subsequently evaporated by a flow of nitrogen at 4 °C forming a thin layer on the walls of a glass tube. Then 1.5 ml of solution containing 15 mM ANTS, 45 mM DPX, 50 mM Tris–HCl, 150 mM NaCl, pH = 7.4 was added and multilamellar liposomes were formed by vigorous shaking of the tube. Large unilamellar vesicles LUV_{100}, LUV_{400} and LUV_{1000} were prepared by repeated extrusion of multilamellar vesicles using the LiposoFast Basic apparatus (Avestin, Canada) with a polycarbonate membrane of appropriate pore diameter (Avestin), as previously described [38]. Liposomes were separated from unentrapped fluorescence dye by gel filtration on a 20 ml column filled with Sephadex G-25M equilibrated with buffer TBS-Ca^{2+} (50 mM Tris–HCl, 150 mM NaCl and 2 mM $CaCl_2$, pH = 7.4). Fractions with a higher content of entrapped dye and quencher were put together and dissolved in TBS-Ca^{2+} to give the final phospholipid concentration of 0.1 mM. This stock solution was stored at 4 °C for no longer than 2 days.

2.3. Leakage of vesicles

A leakage of the vesicles was started by adding a toxin solution (toxin in 8 M urea, maximal volume 40 μl) into 1.6 ml stock solution of liposomes in TBS-Ca^{2+} (see above) followed by incubation in the dark for 3 h at 25 °C. Fast effective mixing immediately after peptide addition was necessary to avoid artifacts caused by transient high local peptide concentration. The protein concentration (CyaA or HlyA) was varied from 0.3 to 80 nM. The final concentration of urea was kept constant at 200 mM. PolyB was used in higher final concentrations from 0.06 to 80 μM. After incubation, 1.5 ml of the suspension was measured in the fluorometer and fluorescence intensity F was recorded (F, actual fluorescence intensity, see Ladokhin et al. for the detailed description of all symbols used in the requenching method [21]). Then DPX (40 μl, 160 mM) was added only once into each sample for the determination of the total quenching Q_{total}. Successive additions of DPX in this case resulted in artifacts since there was a fast back influx of DPX into the vesicles especially if treated with CyaA. At the end of every experiment LUV suspensions were disrupted by the

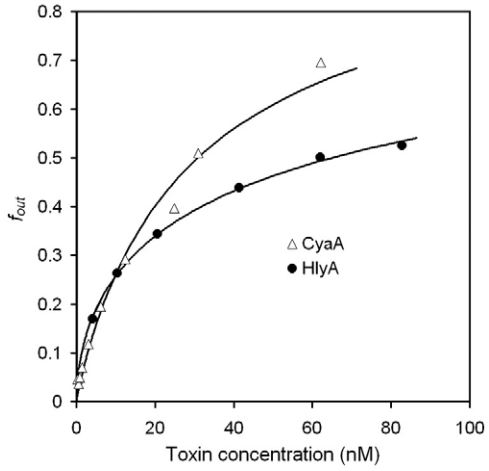

Fig. 2. Leakage of the fluorescence probe ANTS from LUV_{1000} induced by bacterial RTX toxins CyaA and HlyA. The leakage exhibited no positive cooperativity. Concentration dependence of ANTS leakage (f_{out}) showed Hill coefficients $n_{CyaA} = 0.92 \pm 0.06$ for CyaA and $n_{HlyA} = 0.57 \pm 0.02$ for HlyA. Similar values were obtained for liposomes of different size LUV_{100} and LUV_{400} and for DPX release (not shown).

addition of 10 µl of Triton X-100 (10% v/v) for determination of the second value of Q_{total} which allowed calculation of quenching inside LUVs (Q_{in}) and the fraction of ANTS outside (f_{out}). Maximal value of fluorescence intensity F_{max} was obtained after addition of Triton X-100 to untreated vesicles. Subsequent addition of DPX (40 µl, 160 mM) allowed the characterization of quenching of fluorescence outside vesicles Q_{out} (see [21]). Urea (final concentration 200 mM) was used as a blank sample for HlyA and CyaA experiments whereas TBS was used for PolyB in every leakage run. Leakage runs were measured with at least two independent toxin purifications in case of CyaA, CyaA-E509K + E516K and HlyA and were found to be highly reproducible.

2.4. Fluorescence spectroscopy measurement

Fluorescence measurements were performed at 25 °C using FluoroMax-3 (Jobin Yvon, Horriba) fluorometer. Excitation and emission wavelengths were 370 nm and 505 nm, respectively (both bandwidths of 4 nm). Suspension of vesicles (1.5 ml) was placed into 1 × 1 cm quartz cuvette and recorded fluorescence intensities were corrected for background (vesicles without ANTS and DPX, about 2% of total intensity) and for dilution due to addition of the toxins, urea, DPX and Triton X-100.

2.5. Mathematical analysis

Fitting of the experimental data was done with Origin 7.5-based software FluorEssence 2.0 (Horriba Jobin Yvon) using Levenberg–Marquardt iterations without weighting. Error of parameters was estimated using bootstrap analysis. The following form of Hill function was used for fitting our data:

$$f_{out} = \frac{[tox]^n}{K_{50}^n + [tox]^n} \quad (1)$$

where f_{out} is the fraction of ANTS outside vesicles, [tox] is the concentration of the toxin, K_{50} is the concentration of the toxin necessary for the half-maximum leakage and n is the Hill number.

2.6. Mechanism of leakage — requenching method

There are two possible simplified mechanisms of a vesicle leakage — a graded process in which each vesicle releases some part of its contents or an all-or-none mechanism in which some of vesicles release all of their contents and others remain intact. The mechanisms of release of LUV vesicles content were determined using the fluorescence requenching method [21,22] based on the following assumptions: a) when DPX is added into the vesicle suspension, the populations of ANTS molecules inside and outside the vesicles display different susceptibilities to DPX quenching; b) DPX which leaked from vesicles is so diluted that it does not contribute to the quenching of ANTS in solution [21]. Briefly, using this method one measures the dependence of ANTS quenching inside the vesicles Q_{in} (100% quenching corresponds to $Q_{in} = 0$) as a function of the fraction of ANTS that has leaked out of the vesicles f_{out}. In our experiments, this external fraction f_{out} was changed by alternating the amount of toxin added to vesicles whereas the incubation period remained constant.

If Q_{in} observed for different toxin concentrations is independent of f_{out}, then the leakage is interpreted as all-or-none. If Q_{in} increases with f_{out} then the leakage is considered to be graded. For the graded release, Q_{in} depends on f_{out} as follows [21]:

$$Q_{in} = \left\{ \left[1 + K_D \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha}\right] \cdot \left[1 + K_S \cdot [DPX]_0 \cdot (1 - f_{out})^{\alpha}\right] \right\}^{-1} \quad (2)$$

where $[DPX]_0$ is the initial concentration of DPX in the vesicles and α is the selectivity defined as the ratio of the rates of release of DPX and ANTS. The constant K_D is the dynamic quenching constant and K_S is the static quenching constant for the pair ANTS and DPX. In our experiments we found the following values for K_D and K_S: $60 \pm 5\ M^{-1}$ and $115 \pm 10\ M^{-1}$, respectively. Fitting Eq. (2) to the experimental data using non-linear least squares methods gives parameter α, a selectivity of a leakage for given protein. Using this approach it was found for many preparations of vesicles loaded with ANTS and DPX that about 20% of the dye ANTS was outside the vesicles before toxin addition. Although the same osmotic conditions were carefully maintained, there was also some spontaneous decrease of DPX concentration inside the vesicles during their preparation. We also tested the nonreleasable fraction f_{NR}, the amount of ANTS entrapped in multilamellar vesicles, (see [21]). However, the fraction f_{NR} was found to be negligible and this parameter has not been taken into account in this work.

3. Results

3.1. Pores formed by both RTX toxins CyaA and HlyA showed pseudo-first-order kinetics of a leakage

We tested the lytic activities of CyaA and HlyA toxins on LUV_{1000} by varying toxin concentration and leaving both the lipid concentration and incubation time constant (see Material and methods). Both CyaA and HlyA induced efficient escape of ANTS and DPX from LUV_{1000}. Our leakage kinetics data for HlyA and CyaA were comparable with those reported previously [3,4,19]. Fig. 2 shows the fraction of ANTS outside LUVs (f_{out}) as a function of toxin concentration. We fitted our data by Hill equation (Eq. (1)) with Hill coefficients $n_{CyaA} = 0.92 \pm 0.06$ and $n_{HlyA} = 0.57 \pm 0.02$ for CyaA and HlyA, respectively. Using this simplified approach, no positive cooperativity in toxin action was observed implying there was no oligomer formation involved at the stage of LUV membrane permeabilization within the range of toxins concentrations represented on Fig. 2. We also calculated the fraction of DPX which leaked outside ($f\ DPX_{out}$) according to the recently described requenching method [21] and these $f\ DPX_{out}$ data were fitted with Hill function with similar results in terms of cooperativity. Hill coefficients $n \leq 1$ were found for both CyaA and HlyA (not shown).

3.2. CyaA induced a highly cation-selective leakage, while HlyA and PolyB induced a leakage with a low selectivity α

We tested the properties of CyaA pores on LUV in terms of cation selectivity using the requenching method (see Material and

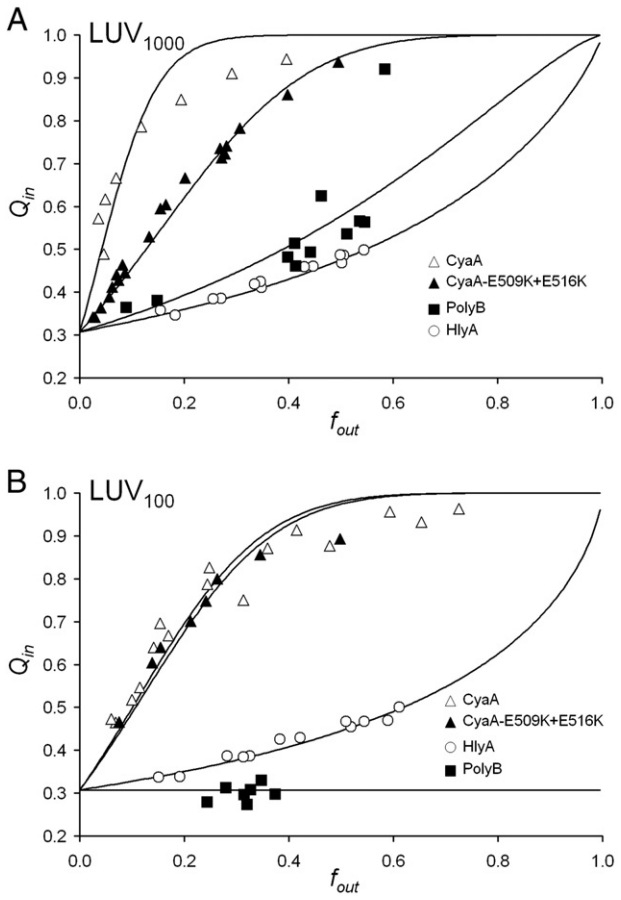

Fig. 3. Mode of action and pore selectivity of different toxins CyaA, HlyA and PolyB on LUV_{1000} and LUV_{100} vesicles. Toxin concentration was varied from 0.3 to 80 nM, lipid concentration was kept constant at 0.1 mM (see Section 2.3 Leakage of vesicles). (A) Experiments with LUV_{1000}. Pore selectivity of CyaA was much higher ($\alpha \sim 15.70 \pm 2.01$) compared with its mutated form CyaA-E509K + E516K ($\alpha \sim 4.97 \pm 0.12$), with substitutions within a predicted amphipathic α-helix. Mode of action of HlyA and PolyB was very similar, graded, and non-preferential ($\alpha = 0.80 \pm 0.01$ and $\alpha = 1.37 \pm 0.17$ for HlyA and PolyB, respectively). (B) Experiments with LUV_{100}. The data of wild type CyaA and CyaA-E509K + E516K overlapped and exhibited in both cases a graded leakage with a higher selectivity for DPX ($\alpha = 6.35 \pm 0.40$ and $\alpha = 5.98 \pm 0.31$, respectively). In contrast, the data of HlyA and PolyB were better resolved showing a non-preferential leakage for HlyA ($\alpha = 0.65 \pm 0.02$) and an all-or-none leakage ($\alpha \sim 0$) for PolyB.

methods for details). CyaA caused a graded leakage of LUV_{1000} encapsulated material with high selectivity for DPX ($\alpha = 15.70 \pm 2.01$) when compared to ANTS (Fig. 3A). A significantly different α value was found for the mutated form CyaA-E509K + E516K ($\alpha = 4.97 \pm 0.12$, Fig. 3A) which was previously reported to form membrane channels with about five times (2.3 versus 10.8) reduced ion selectivity for K^+ versus Cl^- compared to CyaA [15]. This finding was not surprising because, similarly as in the case of K^+ and Cl^- ions, ANTS and DPX molecules can also be considered as an anion/cation pair as was shown in our simple electrophoretic mobility experiment (Fig. 4). When LUV_{100} with much smaller diameter were used to study the selectivity difference between CyaA and its CyaA-E509K + E516K mutated form, almost no difference between their selectivities was observed ($\alpha = 6.35 \pm 0.40$ and $\alpha = 5.98 \pm 0.31$, respectively, see Fig. 3B). Note that the fit of the CyaA data in Fig. 3A did no match the data well, especially at higher toxin concentrations (and higher f_{out}) where the Q_{in} values are located below the fitting curve. This could be attributed to a putative change in pore characteristics at high toxin doses. A similar effect, namely a change in release mechanism after membrane protein aggregation, has already been described [31].

We compared the type of leakage and corresponding selectivities of CyaA toxin and its mutant form CyaA-E509K + E516K with that of HlyA, a related RTX toxin from *E. coli*. HlyA induced a non-preferential graded leakage on both LUV_{100} and LUV_{1000} vesicles ($\alpha = 0.65 \pm 0.02$ and $\alpha = 0.80 \pm 0.01$, respectively, Fig. 3). Values of $\alpha < 1$ can be in fact explained in two ways: i) an improbably high selectivity for ANTS or ii) a combination of an all-or-none and non-selective graded leakage [21]. Rather than a higher selectivity of leakage for ANTS (as could be expected from $\alpha < 1$) we explain this result as a combination of all-or-none and non-selective graded leakage since an all-or-none mechanism for HlyA has already been found by Ostolaza et al. [3] who used the different approach of Parente et al. [39] for the determination of leakage mechanism. When we used PolyB toxin at an extremely high peptide:lipid ratio (up to 1:40) we expected a more dramatic effect on LUV_{100} and LUV_{1000} in comparison with CyaA and HlyA RTX toxins (Fig. 3). PolyB clearly induced an all-or-none leakage of LUV_{100} ($\alpha \sim 0$) and a non-selective graded leakage of LUV_{1000} ($\alpha = 1.37 \pm 0.17$).

3.3. Large LUV_{1000} were more appropriate for evaluation of alpha parameters while small LUV_{100} were more suitable for determination of leakage mechanism

Our data clearly indicated that the LUV mean size markedly affected our results in terms of both selectivity (α value) and the mechanism of leakage, i.e. graded vs. all-or-none leakage. To examine how the LUV diameter alone affects the resulting leakage mechanism of the toxins CyaA, HlyA and PolyB, we complemented previous results with the data obtained using LUV_{400}. The data shown in Fig. 5 indicate that large unilamellar vesicles (LUV_{1000}) were more suitable for distinguishing between high α values (i.e. for different selectivities exhibited by CyaA and CyaA-E509K + E516K) whereas smallest ones

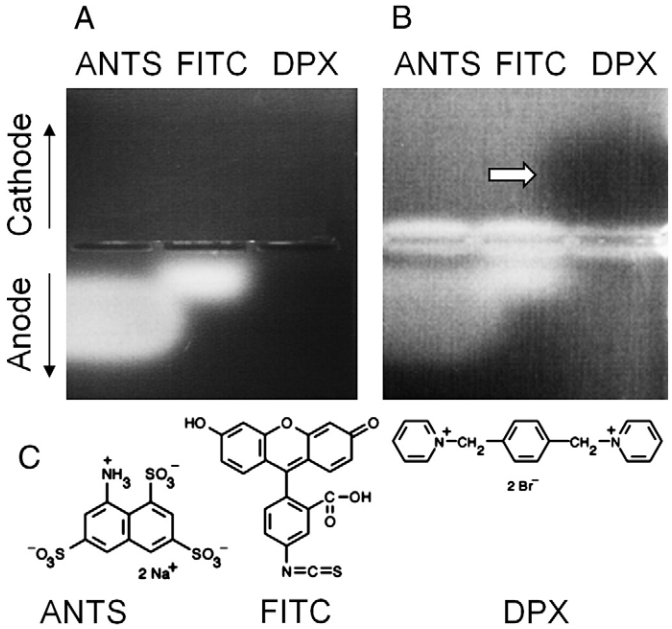

Fig. 4. Agarose gel electrophoresis of fluorescent probes ANTS (50 mM), FITC (5 mM) and the quencher DPX (1 M) under UV illumination, without (A) and with (B) staining with ANTS. Additional staining with ANTS was used to visualize the mobility of quencher DPX. (A) ANTS showed higher mobility toward the positive electrode than FITC. Such FITC behavior might be explained by a different net charge, hydrophobic character of the probe and/or formation of FITC aggregates. (B) After staining of the gel with 50 mM ANTS, quencher DPX was observed as a quenched dark band moving to negative electrode (arrow). Diffusion rates of ANTS and DPX look similar. Agarose gel electrophoresis (2% agarose) was carried out in Tris–HCl 50 mM, NaCl 150 mM, pH = 8, at 5 V/cm for 20 min.

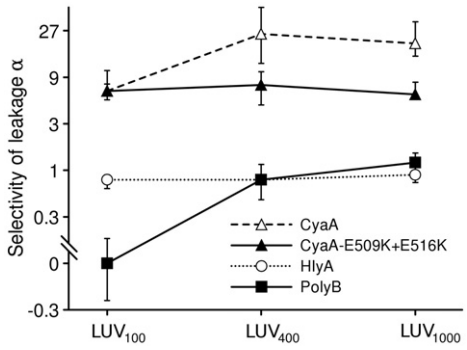

Fig. 5. Selectivity of the leakage of ANTS and DPX from LUV induced by CyaA, CyaA-E509K + E516K, HlyA and PolyB as a function of vesicle size. LUV_{1000} were found to be more suitable for distinguishing between high values of α (CyaA vs. CyaA-E509K + E516K). Smaller LUV_{100}, on the other hand, allowed a discrimination between an all-or-none leakage ($\alpha = 0$) and the graded leakage with low values of α (HlyA vs. PolyB).

(LUV_{100}) allowed us to discriminate the all-or-none leakage ($\alpha = 0$) from the graded leakage with low values of $\alpha > 0$ (HlyA vs. PolyB).

4. Discussion

In our work we studied the mechanism of disruption of large unilamellar vesicles of different sizes (LUV_{100}, LUV_{400}, LUV_{1000}) by two bacterial RTX toxins — CyaA and HlyA and by polymyxin B, a toxin with well-known characteristics of vesicle disruption. Using a requenching method [21] we were able to study the cooperativity of possible toxin oligomers, type of leakage of LUV inner contents (graded or all-or-none leakage) induced by these toxins and selectivity of the toxin channels for anions/cations.

We used a very simplified approach for studying toxin cooperativity, namely the Hill function that is used very often for this purpose. The main disadvantage of the Hill function rests in the fact that it does not describe the nature of the pore formation and the vesicle leakage. In general, one can conclude that toxin oligomers are involved in lysis only when observing sigmoidal dependence of vesicle leakage on toxin concentration. However, data for CyaA or HlyA presented in Fig. 2 indicated that this was not the case. There are other models describing toxin oligomerization and toxin-induced vesicle leakage but they usually expect an a priori all-or-none leakage mechanism. Due to this assumption we could not use such models since in the case of RTX toxins we observed a graded leakage of the vesicle contents.

A recent model of CyaA interaction with membrane suggested that hemolytic and translocation activities are based on the equilibrium between two conformational isomers — oligomers with hemolytic activity and monomers with AC domain translocating activity [15]. We expect that pores formed by CyaA on liposomes are comparable to those observed by these authors since we observed reasonable changes in channel ion selectivity when we used a mutated form of CyaA (CyaA-E509K + E516K) with two substitutions within a predicted transmembrane segment. This mutant toxin exhibited a lowered selectivity of leakage α (leakage of DPX^+ compared with that of $ANTS^-$) induced by the toxin on LUV_{1000}. This finding was in accordance with the modified channel properties of CyaA-E509K + E516K observed on BLM, namely with a reduced ion selectivity for K^+ versus Cl^- compared to CyaA [15,40]. The prolonged lifetime of the mutated channel observed by these authors could be also partially responsible for the differences in α values between CyaA and its mutant observed in our experiments. Such effect of lifetime on α values could be expected according to the model described elsewhere [41]. The authors modeled different modes of dye efflux while varying the ratio of theoretical pore lifetime and vesicle retention time. They predicted the relationship between vesicle diameter and the time necessary for the leakage of vesicle content by diffusion through water-filled pore. According to their model, one could in general expect lower α values for vesicles with smaller diameter. In our experiments, such an effect was observed for CyaA but not for CyaA-E509K + E516K mutant that showed almost a constant value of α independently of vesicle diameter (Fig. 5). We suggest that the successive vesicle leakage with relatively high DPX^+ selectivity induced the formation of membrane potential on LUVs with CyaA, the effect already observed with HlyA [42]. Formation of membrane potential would subsequently lead to a transient slow-down of the DPX efflux thus reducing the α value mainly on larger liposomes LUV_{1000}. Such decrease of α due to the membrane potential formed could also be more pronounced in case of pores with a longer lifetime (CyaA-E509K + E516K). This hypothesis has to be, however, further tested e.g. with some fluorescent probe sensitive to membrane potential.

Mašín et al. proposed recently that CyaA caused the leakage of FITC from LUVs by large-scale membrane disruptions since the rate of FITC release was similar to that of FITC-labeled dextrans [33]. The composition of LUV and other conditions of leakage experiments were the same as the ones used in our work. Their finding, however, contrasts with our results and we therefore compared the efficiency of FITC and ANTS/DPX release induced by CyaA. We found that for a comparable leakage of the probe one needs about 10× lower toxin concentration in case of ANTS/DPX compared to FITC (not shown). In order to show the net charges of ANTS and DPX we performed a semi-quantitative electrophoretic analysis of the charge and diffusion rate of ANTS, FITC and quencher DPX (Fig. 4). We confirmed our prediction that the quencher DPX is positively charged and both dyes ANTS and FITC behave as anions in our experimental conditions. The mobility of FITC was clearly the lowest in all directions, i.e. its electrophoretic behavior was affected not only by its different charge but also by its molecular shape and size (Fig. 4C). The most probable explanation of a slow FITC leakage besides the low permeability of CyaA toxin pores for anions is therefore the formation of poorly soluble aggregates of FITC in liposomes that can be released from LUVs only at high toxin concentration able to disintegrate the membrane. CyaA channels were found to be permeable for solutes with molecular size comparable or smaller than that of FITC, ANTS or DPX [13]. Therefore, the leakage of FITC-labeled dextrans [33] must be interpreted as some large-scale membrane perturbation that was also able to release FITC aggregates. Aggregation of fluorescein based probes was already reported [43].

In experiments with another RTX toxin, HlyA, we found a pseudo-first-order leakage with low selectivity α. Such lack of cooperativity of a dye leakage induced by HlyA was also observed previously [4]. Contradictory results in terms of HlyA cooperativity were obtained on BLM; Menestrina et al. found linear dependence of the membrane conductance on HlyA concentration [44] whereas other authors observed a steep curve with a slope ~3 in a double logarithmic plot [14]. For low doses of the toxins the value of this slope should correspond to a Hill number. However, we concluded that more sophisticated model for description of pore formation must probably be used to yield relevant outcome.

An all-or-none leakage of LUVs by HlyA was reported elsewhere [3]. Their result together with our finding (non-selective graded leakage) could be compatible with the formation of large channels with long lifetimes observed by other authors on BLM [14]. On the other hand, the reported all-or-none mechanism mentioned above is in contrast with the data published by other authors [42] who suggested that HlyA formed cation-selective pores directly generating electric membrane potential on the membrane. Such effect is clearly compatible only with a graded leakage.

Our results show that the pore-forming properties of both RTX toxins, CyaA and HlyA can be effectively compared using the requenching method with a set of LUV of different diameters. The differences in crucial characteristics of the pores formed by toxins, i.e. the pore size or ion selectivity, are both manifested in the differences

of parameter α. The effects of pore lifetime and vesicle size on parameter α have to be studied in more detail in order to develop a new model of vesicle leakage that would include these parameters. Such improvement of the model would greatly enhance the ability of the fluorescence requenching method to describe the interaction between the protein toxin and biological membrane.

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